From DEPARTMENT OF BIOSCIENCES AND NUTRITION

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MODULATION OF NUCLEAR RECEPTOR SIGNALING BY RBR UBIQUITIN LIGASES

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To my family

ABSTRACT

Nuclear receptors (NRs) constitute a superfamily of transcription factors and play important roles in physiology. Transcriptional regulation by NRs can be modulated through interactions with various coregulators that activate or repress transcription through mediating receptor and chromatin modifications as well as communicating with the general transcription factor machinery. Coregulators also affect NR protein stability, which led to the discovery that the ubiquitin-proteasome system regulates transcriptional activity of certain NRs. However, only a few E3 ubiquitin ligases, that mediate the substrate specificity in the ubiquitin-proteasome system, have been identified as NR coregulators. The overall aim of this thesis was to identify novel RING-in-between-RING (RBR) E3 ubiquitin ligases that modulate NR signaling.

In the first study of this thesis, we provide evidence that the RBR ubiquitin ligase RNF31 acts as a novel coregulator for the NR DAX-1 in steroidogenesis. We demonstrate that RNF31 interacts with, and monoubiquitinates, DAX-1 and maintains DAX-1 stability. RNF31 is necessary for the formation of a ternary corepressor complex of RNF31, DAX-1 and SMRT on the DAX-1 target gene promoters CYP19 and Steroid Acute Regulatory protein.

In the second study, we identify the RBR ubiquitin ligase RBCK1 to be a novel cell cycle regulator in breast cancer cells through modulating expression of the cell cycle regulators Cyclin B1 and Estrogen Receptor α (ERα). We demonstrate recruitment of RBCK1 to the breast-cancer associated ERα promoter B and find in several independent studies that RBCK1 mRNA correlates with ERα mRNA expression in breast cancer.

In the third study, we demonstrate that RBCK1 interacts with ERα and enhances ERα transcriptional activation of its own promoter. Further, we show occupancy of the RBCK1-interacting protein Protein Kinase C beta 1 ($PKC\beta_I$) at the ERa promoter B. Consistent with this, $PKC\beta_I$ modulates ERα expression. A ternary complex of ERα, RBCK1 and $PKC\beta_I$ on the ERα promoter B correlates with histone modifications associated with a permissive chromatin environment. Taken together, the two final studies suggest an ERα coactivator function of RBCK1 at the ERα promoter.

In conclusion, the papers included in this thesis demonstrate that the RBR ubiquitin ligases RNF31 and RBCK1 are novel NR-interacting proteins that modulate NR-dependent transcription through non-proteolytic coregulatory functions. Both the ligases are recruited to receptor target gene promoters and are necessary for formation of transcriptional complexes associated with repression and activation, respectively. These findings clearly support a coregulatory function of E3 ubiquitin ligases in NR signaling beyond degradation.

LIST OF PUBLICATIONS

I. Ehrlund A, Holter Anthonisen E, **Gustafsson N**, Venteclef N, Robertson Remen K, Damdimopoulos AE, Galeeva A, Pelto-Huikko M, Lalli E, Steffensen KR, Gustafsson JÅ, Treuter E. *E3 Ubiquitin Ligase RNF31 Cooperates with DAX-1 in Transcriptional Repression of Steroidogenesis.* Mol Cell Biol. 2009 Apr;29(8):2230-42. Epub 2009 Feb 23.

II. **Gustafsson N**, Zhao C, Gustafsson JÅ, Dahlman-Wright K. *RBCK1 drives breast cancer cell proliferation by promoting transcription of Estrogen Receptor-alpha and Cyclin B1.* Cancer Research 70, 1265, February 1, 2010. Published Online First January 26, 2010; doi: 10.1158/0008-5472.CAN-09-2674

III. **Gustafsson Sheppard N**, Dahlman-Wright K. *Estrogen receptor-alpha, RBCK1 and protein kinase C beta1 cooperate to regulate estrogen receptor-alpha gene expression.* Manuscript

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LIST OF ABBREVIATIONS

AF	Activating function
AHC	Adrenal Hypoplasia Congenita
AI	Aromatase Inhibitors
AP-1	Activating Protein 1
AR	Androgen Receptor
ChIP	Chromatin Immunoprecipitation
CHIP	Carboxyl Terminus of Hsc70-interacting Protein
DAX-1	DSSR, AHC, critical region on the X-chromosome, gene 1
DBD	DNA-binding domain
DSSR	Dosage-sensitive sex reversal
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen Receptor
ERE	Estrogen Response Element
GTF	General Transcription Factor
H12	Helix 12
H3	Histone 3
HECT	Homologous to E6-AP
HER2	Human Epidermal Growth Factor Receptor 2
HRE	Hormone Response Element
LBD	Ligand-binding domain
MDM2	Murine double minute clone 2
NCoR	Nuclear Receptor CoRepressor
NR	Nuclear Receptor
PR	Progesterone Receptor
PKC	Protein Kinase C
RBCK1	RBCC protein interacting with PKC 1
RBR	RING-In-Between-RINGs
RING	Really-Interesting-New-Gene
RNF31	RING finger 31
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulators
SF-1	Steroidogenic Factor-1
StAR	Steroid Acute Regulatory Protein
SMRT	Silencing Mediator of Retinoid and Thyroid Receptors
SRC	Steroid Receptor Coactivator
Ub	Ubiquitin

1 POPULAR SCIENTIFIC SUMMARY IN SWEDISH

1.1 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kärnreceptorer är mottagare i våra celler som reglerar viktiga fysiologiska funktioner. Receptorerna reglerar cellulära funktioner genom att binda till specifika regulatoriska regioner i vår arvsmassa och därmed bestämma om kodande delar av arvsmassan, så kallade gener, ska aktiveras eller inaktiveras. Detta leder till ökade eller minskade nivåer av proteiner i våra celler och påverkan på cellulära funktioner. Syftet med denna avhandling har varit att bestämma hur förmågan hos kärnreceptorer att aktivera eller inaktivera gener påverkas av en specifik typ av enzymer, så kallade ubiquitin ligaser.

Ubiquitin ligaser är enzymer som sätter ett litet protein kallat ubiquitin på andra proteiner, denna process kallas ubiquitinering. Från början trodde man att ubiquitinering endast ledde till nedbrytning av proteinet dock har man det senaste decenniet påvisat att ubiquitin ligaser kan ha regulatoriska egenskaper vid aktivering av gener. Man har identifierat att ett fåtal av dessa ubiquitin ligaser binder till kärnreceptorer och påverkar deras förmåga att reglera genaktivitet.

Studie I: I binjuren finns en kärnreceptor kallad DAX-1 som påverkar binjurefunktion genom att reglera produktionen av hormoner. DAX-1 binder till andra kärnreceptorer och motverkar deras aktivering av gener. Hitintills vet man inte hur DAX-1 regleras eller aktiveras. I denna studie identifierade vi att ubiquitin ligaset RNF31 binder till DAX-1 och ökar förmågan hos DAX-1 att motverka aktivering av gener viktiga för hormonproduktion.

Studie II och III: Östrogen är ett hormon som binder till specifika kärnreceptorer, östrogenreceptor α och β, i våra celler. När östrogen binder till östrogenreceptor α blir receptorn aktiv i cellen och binder till specifika regioner i vår arvmassa vilket leder till ökade nivåer av proteiner som stimulerar celltillväxt. I utveckling av bröstcancer ökar den intracellulära mängden östrogenreceptor α och celltillväxt sker ohämmat. Ökade nivåer av receptorn är en stark riskfaktor för utveckling av bröstcancer och användning av läkemedel som motverkar bindning av östrogen till receptorn är vanlig behandling för bröstcancerpatienter. Dock förekommer resistans till sådan behandling, därför är det viktigt att öka vår förståelse för hur östrogenreceptor α kan regleras.

I studie II identifierade vi att nivån av östrogenreceptor α i bröstcancerceller påverkas av ubiquitin ligaset RBCK1. När vi tog bort RBCK1 från bröstcancercellerna minskade nivån av receptorn och celltillväxten hämmades. Vi observerade att RBCK1 binder till specifika regulatoriska regioner i vår arvsmassa som aktiverar genen för östrogenreceptor α.

I studie III karaktäriserade vi hur RBCK1 reglerar aktiviteten av genen för östrogenreceptor α i bröstcancerceller. Vi identifierade att RBCK1 tillsammans med östrogenreceptor α och protein kinas C β 1 (PKCβ1) binder till arvsmassan vilket leder till kemiska förändringar i arvsmassan som stimulerar aktivering av genen för östrogenreceptor α. Genom att motverka PKCβ1 kunde vi minska den intracellulära nivån av receptorn.

Vår förhoppning är studierna i denna avhandling kan hjälpa till att identifiera nya tillvägagångssätt för att behandla sjukdomar som uppkommer på grund av ohämmad reglering av kärnreceptorerna DAX-1 och östrogenreceptor α.

2 INTRODUCTION

2.1 NUCLEAR RECEPTORS

Nuclear receptors (NRs) constitute a superfamily of transcription factors that play a central role in diverse physiological functions such as metabolism, reproduction and development. Their importance is highlighted by the fact that NRs are common pharmacological targets in inflammation-related diseases and cancer. In humans, 48 NRs have been identified. The receptors are structurally and functionally related, illustrated by their modular structure that includes six domains: a variable N-terminal domain A/B, a conserved DNA-binding domain C (DBD), a non-conserved hinge domain D, a C-terminal ligand binding domain E (LBD) and a variable F domain [1,2]. The receptors can be divided into subfamilies based on their sequence homology, see Figure 1 [2].

Small molecule substances (ligands) that bind specifically to the LBDs have been identified for 24 of the 48 NRs, while for the remaining 24, potential ligands have not yet been identified and are therefore classified as orphan receptors. NR ligands show large diversity in their structures, even though the receptors have a conserved structure for the LBD. The LBD consists of 12 α-helixes, with crystal structures revealing that the LBD undergoes a conformational change upon binding to ligands, involving repositioning of helix 12 (H12), which converts the receptor into an active holo-conformation [3,4]. H12 is part of the activating function-2 (AF-2) domain and it is the repositioning of this helix upon ligand-binding that enables interaction with transcriptional coregulators and subsequently activation or repression of gene expression [5]. Additionally, the LBD and D domain both contain nuclear localization signals, and the LBD and DBD dimerization regions for receptors that dimerize. Depending on the identity of a given NR, the receptors form monomers, homodimers or heterodimers prior to DNA-binding and regulating transcription. Another activation function, AF-1, resides in the A/B domain. AF-1 is generally thought to mediate ligand-independent activation of the receptors.

Recognition and regulation of target genes occur via hormone response elements (HRE) in the DNA. The DBDs of the NRs directly contact the HRE, for which the consensus sequence is RGGTCA and depending on the type of receptor the sequence can be modified, for example, inverted or extended [6].

Figure.1 *Members of the human Nuclear Receptor Superfamily*

SUBGROUP 1	
Name	**Abbreviation**
Thyroid Hormone Receptor	TRα TRβ
Retinoic Acid Receptor	RARα RARβ RARγ
Peroxisome Proliferator-Activated Receptor	PPARα PPARβ PPARγ
Reverse erbA	Rev-erbα Rev-erbβ
RAR-related orphan receptor	RORα RORβ RORγ
Liver X Receptor	LXRα LXRβ
Farnesoid X receptor	FXRα FXRβ
Vitamin D Receptor	VDR
Pregnane X Receptor	PXR
Constitutive Androstane Receptor	CAR
SUBGROUP 2	
Name	**Abbreviation**
Human Nuclear Factor 4	HNFα HNFγ
Retnoid X Receptor	RXRα RXRβ RXRγ
Testis Receptor	TR2 TR4
Tailless	TLL
Photo-rReceptor Specific Nuclear Receptor	PNR
Chicken Ovalbumin Upstream Promoter-Transcription Factor	COUP-TF1 COUP-TFII
ErbA2-realted gene-2	EAR2
SUBGROUP 3	
Name	**Abbreviation**
Estrogen Receptor	ERα ERβ
Estrogen-Related Receptor	ERRα ERRβ ERRγ
Glucocorticoid Receptor	GR
Mineralcorticoid Receptor	MR
Progesterone Receptor	PR
Androgen Receptor	AR
SUBGROUP 4	
Name	**Abbreviation**
NFG-Induced Factor B	NGFIB
Nur Related Factor 1	NURR1
Neuron-derived orphan receptor 1	NOR1
SUBGROUP 5	
Name	**Abbreviation**
Steroidogenic Factor 1	SF1
Liver Receptor Homologous Protein I	LRH1
SUBGROUP 6	
Name	**Abbreviation**
Germ Cell Nuclear Factor	GCNF
SUBGROUP 0	
Name	**Abbreviation**
DSS-AHC critical region on the chromosome, gene 1	DAX1
Short Heterodimeric Partner	SHP

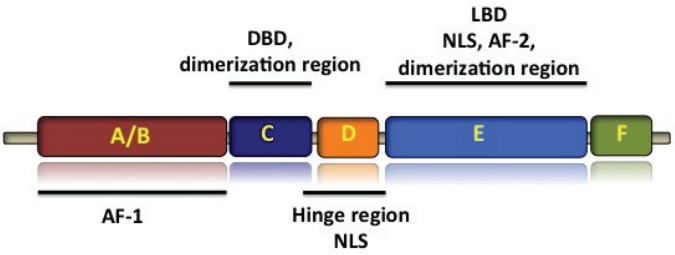

Figure 2. *Modular structure of Nuclear Receptor domains. NLS, nuclear localization signal.*

2.2 ESTROGEN RECEPTORS

In the 1960's Elwood V. Jensen discovered that the action of the steroid hormone estrogen was mediated by a receptor [7] that we today know as Estrogen Receptor α (ERα). ERα was later cloned in 1985 [8]. ERα is widely expressed in the human body including the uterus, liver, kidney, heart, mammary gland, epididymis, thyroid, adrenal, bone, and parts of the brain [9]. A second estrogen receptor, ERβ, was cloned in 1996 [10]. ERα and β belong to the NR3A subgroup of NRs and even though they are encoded by different genes they display a high degree of sequence similarity in the DBD (98%). The sequence similarity in the LBD is 59%, consistent with this they have different affinities for some ligands. ERα and β are coexpressed in some tissues and the receptors can homo- and heterodimerize. Estrogen can also mediate signaling via the Membrane associated G-protein coupled ER (GPR30) [11].

2.3 ESTROGEN RECEPTOR ALPHA

2.3.1 Molecular mechanisms of ERα action

Upon binding to estrogen, ERα regulates the expression of specific target genes that mediate cell proliferation, differentiation and motility. The most well studied function of ERα is its proliferative role in breast cancer [12]. ERα is a highly dynamic protein that

can shuttle between the cytoplasm and nucleus [13], thus it promotes physiological functions via several mechanisms both in the nucleus and cytoplasm, as well in ligand-dependent and ligand-independent fashions [12].

2.3.1.1 Ligand-dependent mechanisms

In its unliganded state, ERα is part of a multiprotein complex containing heat-shock proteins such as Hsp90[14]. The conformational change in ERα upon binding to estrogen [15] allows the dissociation of ERα from the complex, then dimerization of the receptor[16] [17], and subsequent binding to estrogen response elements (EREs) in the DNA. Finally, recruitment of coactivators and GTFs occur to form a stable preinitiation complex leading to the activation of target genes. Upon binding to estrogen, H12 closes over the LBD cavity enabling interaction with coactivators via the AF-2 domain. [15]. The AF-1 domain also takes part in the ligand-dependent transcriptional activation of ERα via recruitment of coactivators. Depending on promoter and cell type, the AF-1 and AF-2 domains may cooperate or act independently in ligand-dependent regulation of ERα target gene transcription[18,19].

Upon estrogen stimulation, ERα can also regulate transcription at non-ERE sites via indirect binding to DNA involving interaction with other transcription factors such as Activating Protein 1(Ap1) [20], Specificity Protein 1 (Sp1) [21] or nuclear factor kB (NFkB) [22].

2.3.1.2 Ligand-independent mechanism

ERα can also become activated in a ligand-independent manner. Such an example is receptor tyrosine kinase signaling, mediated by epidermal growth factor and insulin-like growth factor, that triggers phosphorylation of ERα in the A/B domain through the Ras-MAPK pathway in the absence of estrogen [23-25]. This phosphorylation event promotes interaction with co-activators and subsequently transcriptional activation [26]. Also phosphorylation of ERα by Protein Kinase A has been described to lead to transcriptional activation by ERα [27].

2.3.1.3 Non-genomic ERα signaling

Recently, methylation of ERα was linked to transcriptional activation independent of binding to DNA by ERα. Stimulation by estrogen induces methylation in the DBD

leading to formation of complex with PI3K, Src and focal adhesion kinase. Activating Akt signaling subsequently leads to initiation of transcription and cell proliferation [28]. Thus, ERα can influence gene expression indirectly.

2.3.2 Regulation of ERα activity by ligands

2.3.2.1 Estrogen

The natural ligand for ERα is the steroid hormone estrogen. There are 3 different forms of estrogen; estradiol (E2), estriol (E3) and estrone (E1). E2 has the highest affinity for ERα and is the predominant form of estrogen in premenopausal women while E1 is mainly produced in postmenopausal women. E2 and E1 are produced mainly in the ovaries in premenopausal women, while adipose tissue is the main producer of these estrogens in postmenopausal women. E3 is the predominant form of estrogen in pregnant women where it is produced by the placenta. [29] [30].

2.3.2.2 Phytoestrogens

In addition to endogenous estrogen, plant-derived compounds that have a similar structure as estrogen, so called phytoestrogens, can also bind to ERα and exert antagonistic and agonistic properties [31].

2.3.2.3 Selective Estrogen Receptor Modulators,

A major effort by pharmaceutical companies has been put into developing synthetic ligands that antagonize ERα action in the breast and uterus, in order to inhibit estrogen-stimulated cancer growth, but display agonistic properties in bone and the cardiovascular system, in order to maintain the beneficial effect of estrogen in these organs[32]. Selective Estrogen Receptor Modulators (SERMs) are compounds that display tissue-selective agonist and antagonistic responses. The basis for the SERM concept was the observation that Tamoxifen works as an ERα antagonist in the breast inhibiting cell growth, but as an ERα agonist in the uterus promoting cell growth [32]. When ERα is bound to Tamoxifen, H12 changes its position away from the LBD cavity, preventing coactivators from interacting with the AF-2 domain [15]. Reasons behind distinct agonist and antagonist profiles in different tissues were originally believed to include the presence and ratio of different nuclear receptor coregulators

interacting via the AF-1 domain and the type of promoter [33,34]. Importantly, it was shown that binding to Tamoxifen reveals a novel binding surface in the LBD for coregulators, independent of AF-2, referred to as AF-T [35,36]. As Tamoxifen promots cell growth in the uterus and resistance to Tamoxifen is a recognized clinical problem in treatment of breast cancer (see section 2.4.1), structurally different SERMs have been developed, including Raloxifene [32].

27-hydroxycholesterol was recently identified as the first endogenously occurring SERM. Whereas 27-hydroxycholesterol inhibits ERα action in the cardiovascular system [37], it stimulates proliferation in breast cancer cell models [38]. As levels of 27-hydroxycholesterol are directly related to cholesterol levels, this could provide an important link between breast cancer and cholesterol levels.

2.3.2.4 Selective Estrogen Receptor Degraders

In contrast to partial antagonists such as Tamoxifen, pure antagonists without any agonistic effects have also been developed. This includes ICI 182,780 (Fulvestrant), which is a Selective Estrogen Receptor Degrader (SERD) with high affinity binding to ERα. Fulvestrant inhibits dimerization of ERα and induces rapid degradation of the receptor by the proteasome.

2.4 BREAST CANCER

Over a century ago, George Thomas Beatson published the discovery that breast cancer patients benefited from the removal of ovaries [39]. As the ovaries produce estrogen in premenopausal women [30], this report was the first to suggest a link between estrogen and breast cancer. Breast cancer is the most common cancer in women in the western world, responsible for approximately 458 400 deaths in the year 2008 [40]. Breast cancer can be classified into the following subgroups based on immunhistochemical staining of ERα, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [41,42];

Luminal, subtype A:	ERα and PR positive, HER-2 negative
Luminal, subtype B:	ERα, PR and HER-2 positive
Her2 overexpression:	ERα and PR negative, HER-2 positive
Basal-like:	ERα, PR and HER-2 negative

2.4.1 ERα and breast cancer

As approximately 70% of all breast cancers are ERα-positive and considered to be dependent on ERα for cell growth, this makes ERα an important therapeutic target in breast cancer [43]. The risk of developing breast cancer is increased in mammary tissues with high ERα expression [44], with ERα-positive benign breast epithelium having a 6 times higher chance to develop into breast cancer compared to ERα-negative benign breast epithelium [45]. This is consistent with the fact that ERα expression may be a cause rather than a consequence in breast cancer development. It is generally thought that, while ERα has a proliferative role upon estrogen binding, ERβ is anti-proliferative and inhibits the transcriptional activity of ERα in breast cancer [9,46].

2.4.1.1 Regulation of ERα expression in breast cancer

The ERα gene contains eight upstream exons, see Figure 2A, with seven alternative promoters displaying tissue selective expression. All upstream exons are spliced to a common acceptor splice site located just after exon A to produce the full-length ERα protein [47]. Generally, tissues with high ERα expression such as ovaries and breast use promoter A, B or C, whereas promoter E and F are commonly used within tissues with low ERα expression such as liver and bone [48-50].

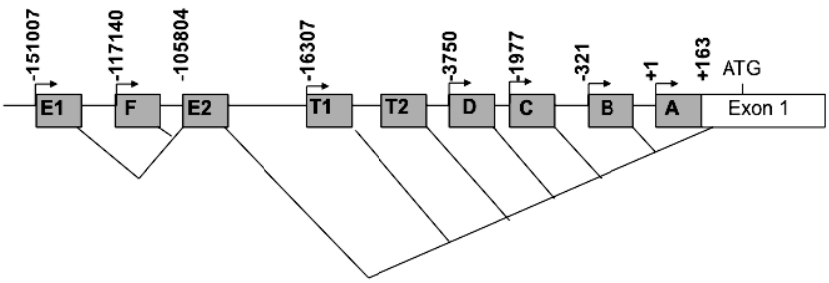

Figure 3. *Structure and organization of ERα promoters and corresponding upstream exons.*

Autoregulation is a common feature among the NR superfamily and ERα is not an exception [6]. ERα binding sites have been reported in the proximal promoters A and B, and in 3 upstream enhancers [51,52]. The possible combined influence of ERα binding sites in upstream enhancers and proximal promoter regions on ERα expression remains to be determined. It is however generally thought that ERα negatively regulates its own expression, as expression of ERα and estrogen levels are inversely correlated under normal physiological conditions in the breast [53]. Consistent with this, recruitment of the corepressor Sin3A by ERα to the proximal ERα promoter upon E2 stimulation has been reported [52]. The inverse relationship between ERα and estrogen is lost in breast cancer [53] and antagonist treatment with Fulvestrant or Tamoxifen leads to downregulation of ERα mRNA [54,55]. Thus, it is possible that ERα positively regulates its own expression in breast cancer. Moreover, high levels of ERα, such as in development of breast cancer, can lead to E2-independent activation of gene expression by ERα [56,57].

ERα gene amplification accounts for altered ERα expression in 50% of ERα-positive breast cancers [58], suggesting that other processes such as regulation of ERα expression or protein stability become deregulated in breast cancer. ERα mRNA containing exon B is overexpressed in breast cancer tissue compared to normal breast tissue and shows high correlation with ERα protein levels in breast cancer tissue [49]. In support of this, studies in breast cancer cell culture show increased ERα promoter B activity [59]. This suggests there may be breast-cancer selective regulation of ERα via promoter B.

A few transcription factors have been reported to regulate ERα gene expression in breast cancer cells. Positive regulation is mediated by the transcription factors Estrogen Receptor Factor 1 (ERF1), Forkhead Box M1 (FoxM1) and the tumour suppressor p53 via association to the proximal ERα promoters [60-62]. BARX2 is a tumour suppressor that activates ERα expression through association to the promoter E and F (Stevens et al 2004, Stevens and Meech 2006). GATA3 binds to an enhancer region between the ERα promoter E and F and recruits RNA polymerase II, activating ERα gene expression [63]. The Wilms' tumour suppressor 1 protein represses ERα expression [64].

2.4.1.2 Treatment of ERα-positive breast cancer

In ERα-positive breast cancer SERMs that display antagonistic properties on ERα signaling in breast, such as Tamoxifen, are standard endocrine treatment for all stages

of breast cancer [65]. About 80% of ERα-positive and PR-positive breast cancers are responsive to Tamoxifen treatment, while in ERα-positive and PR-negative breast cancer only 40% are responsive [66,67]. However, one third of women treated with Tamoxifen for 5 years will have recurrent disease within 15 years [32]**.** Another frequently used SERM is Raloxifene however, as therapy with Raloxifene for 8 years is only effective in 65% of ERα-positive breast cancer, it suggests that acquired resistance to Raloxifene may also occur [68]. An explanation for acquired resistance was long believed to be due to loss of ERα expression. However, as only 15-20% of endocrine resistant breast tumors have loss of ERα expression [69], deregulated ERα signaling or possibly upregulation by alternative proliferative pathways by Tamoxifen or Raloxifene also contributes to resistance. Of those patients with acquired Tamoxifen resistance, 10-20% will benefit from treatment with the pure antagonist Fulvestrant. [70,71].

Aromatase inhibitors (AI) are another treatment for ERα-positive breast cancer. AI specifically targets CYP19 (aromatase), the rate-limiting enzyme that catalyzes the last step in the estrogen synthesis, thus decreasing the production of estrogen. 10-20% of patients with acquired resistance to Tamoxifen will benefit from treatment with aromatase inhibitors [70,71].

2.5 COREGULATORS

The importance of interaction with coregulators for successful transcriptional activation by NRs was initially demonstrated in experiments with point mutations in LBD helices 3, 5 and 12 of ERα. The mutations did not affect interaction with ligands or DNA, however the AF-2 activity was completely abolished, indicating that these helices form a surface needed for contact with additional proteins that we today know as coregulators [72,73]. In 1995 the first nuclear receptor coregulator, steroid receptor coactivator-1 (SRC-1), also known as NCOA1), was cloned [74]. Even though coregulators are widely expressed in the human body, they are believed to be expressed in different ratios depending on the cell type which enables tissue-specific actions by NRs [75]. The functional importance of coregulators are emphasized by the fact that 164 of the currently identified coregulators have been associated to some type of disease [76]. Coregulators can also be regulated by post-transcriptional modifications, adding an additional layer of complexity to gene regulation by NRs [77].

Coregulators can broadly be dived into coactivators or corepressors depending if they enhance or repress transcriptional activation by the receptor. Coactivators were originally thought to interact with agonist or partial antagonist bound receptors with corepressors interacting with the un-liganded or antagonist bound receptor [5,78]. However, studies have shown that ligand-activated ERα can repress transcription [79] by recruitment of corepressors [80].

To date, about 240 coactivators and 40 corepressors have been identified that interact with NRs. Some of these interact with several receptors while some are receptor-specific [81]. Considering the large number of coregulators in relation to the number of NRs, it is generally thought that coregulators form and function in multiprotein complexes [82,83].

2.5.1 Coactivators

Mapping the domain necessary for interaction between coactivators and the AF-2 domain in ligand-bound nuclear receptors, led to the identification of the LXXLL-motif (L is leucine and X is any amino acid, also called NR-box) in coactivators [84]. Specificity regarding the interaction between NRs and coactivators is determined by the amino acid sequence surrounding the LXXLL-motif [85] as well as using different combinations of multiple LXXLL-motifs for interaction with different nuclear receptors [86]. Coactivators that interact with NRs through the AF-1 domain have also been identified [87,88].

Some coactivators directly affect chromatin environment, such as SWI/SNF that mediates ATP-dependent chromatin remodeling and CBP/p300, SRC-1, SRC-2 and SRC-3 that display histone acetyltransferase activity [82].

2.5.2 Corepressors

Corepressors can also interact via LXXLL-motifs with ligand-bound receptors and thus compete with coactivators for binding. Some corepressors contain a specific motif called the CoRNR-box that is an extended LXXLL-motif used to bind to the LBD of their interacting NRs. The most well studied corepressors are Nuclear Receptor

CoRepressor (NCoR) and Silencing Mediator of Retinoid and Thyroid Receptors (SMRT), both of which interact with NRs through their CoRNR-boxes to form large protein complexes containing histone deacetylase activity [89]. Tamoxifen treatment has been suggested to promote interaction with corepressors such as SMRT that in turn inhibits transcription [90,91]. Treatment with glucocorticoid receptor (GR) and PR antagonists also show recruitment of SMRT and NCoR [92]. However, in the case of the androgen receptor (AR), agonist-bound AR interacts with NCoR and SMRT [91]. Thus, the mechanisms by which these corepressors interact with NRs seems to differ depending on type of NR.

2.6 DAX-1

Dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene 1 (DAX-1) is an orphan NR, for which no ligand has been identified, located on the X-chromosome [93]. DAX-1 belongs to the NR0B1 subgroup of NRs and is mainly expressed in testis and adrenals but is also expressed in other tissues such as breast [94] and liver [95,96].

The modular structure of DAX-1 includes an LBD in its C-terminal but lacks the hinge domain and the classical DBD. The N-terminal contains three LXXLL-motifs through which DAX-1 interacts directly with the AF-2 domain in NRs and represses their function, acting as a NR corepressor [97-99]. NRs that DAX-1 function as a corepressor for includes ERα [97], PR, AR [100], peroxisome proliferator-activated receptor γ [101], HNFα [96], liver X receptor α [95], Nur77 [102], steroidogenic factor-1 (SF-1) and liver receptor homolog-1 [99]. DAX-1 can also bind directly to DNA and RNA via the LXXLL-motifs [103,104].

A functional DAX-1 is important for proper steroidogenesis. Deletion of DAX-1 or mutated DAX-1 leads to underdevelopment of the adrenal glands with hypoplasia from birth, and failed production of steroid hormones, whereas duplication of DAX-1 causes dosage-sensitive sex reversal [93,105].

In the adrenal, DAX-1 is expressed in the outer cortex, which is the area responsible for steroid hormone production [93]. DAX-1 inhibits steroidogenesis through repression of

transcription of steroid acute regulatory protein (StAR) and CYP19 (aromatase) [106]. StAR is responsible for transporting cholesterol to the inner mitochondrial membrane, which is the first step in steroid hormone synthesis [107]. Two mechanisms for how DAX-1 exerts its corepressor function have been suggested. Firstly, DAX-1 uses its LXXLL-motifs to compete with coactivators for binding to the AF-2 domain of NRs. In the case for repression of StAR, DAX-1 interacts with SF-1, which is the transcriptional activator for StAR, thereby repressing SF-1 induced activation of StAR [99]. Secondly, DAX-1 represses transcription directly, through binding to the StAR promoter via its C-terminal transcriptional silencing domain [106] [108]. Interestingly, the C-terminal transcriptional silencing domain is deleted in all adrenal hypoplasia congenita (AHC) patients [109].

DAX-1 interacts with other corepressors via its LBD [110,111]. Importantly, mutations in the C-terminal of DAX-1, particularly in helix 12, result in a more cytoplasmic localization of DAX-1 [112]. Thus, loss of interaction between DAX-1 and corepressors due to a change in the intracellular localization may contribute to the development of AHC.

Surprisingly, SF-1 knockout mice display a similar phenotype as patients with DAX-1 mutations [108]. This suggests that DAX-1 and SF-1 cooperate to stimulate steroidogenesis, which appears in contrast to the mechanism of DAX-1 inhibition of SF-1 function described above. Thus, the molecular mechanism of DAX-1 is complex, revealing a need for further investigations.

2.7 UBIQUITINATION

Regulation of protein degradation (proteolysis) is an important process in order to maintain cell homeostasis. Ubiquitination is a process in which ubiquitin (Ub) becomes covalently attached to a protein substrate. In the case of proteolysis this leads to recognition of the Ub modified protein by the 26S proteasome and ultimately degradation of the protein.

Conjugation of Ub to a protein requires three different classes of enzymes that work in a 3-step process: 1) the Ub activating enzyme E1 activates Ub in an ATP-dependent

manner 2) Ub is then transferred to the Ub conjugating enzyme E2 3) the actual ubiquitination takes place when E2 interacts with the Ub ligase E3 that recognizes the protein to be ated. E3s catalyse the formation of an isopeptide bond between a lysine residue in the target protein and a glycine 76 in the C-terminal of Ub, thus it is the combination of E2 and E3 enzymes that determine the substrate specificity [113-115]. In the human genome there exists one E1, approximately 40 E2s and several hundreds of E3s [114,116]. E3s can be divided into 2 families based on their catalytic domain; Homologous to E6-AP (HECT) domain and Really Interesting New Gene (RING) finger [117]. The main difference between the two families is the way in which they transfer Ub to the protein substrate, i.e aminolysis. RING E3s bring the E2-Ub thioester close enough to the substrate in order to transfer Ub, thus the active site is present in the E2. HECT E3s on the other hand contain an active site and transfer Ub directly to the protein substrate.

Ub can either be conjugated to one lysine in the protein substrate, called monoubiquitination, several Ubs can be conjugated to multiple lysine sites in the protein, called multiubiquitination or finally Ubs can form polyubiquitin chains. Ub contains seven lysine residues, all which can conjugate to the glycine 76 in other Ubs in order to form distinct polyubiquitin chains. Such chains can also be formed when the C-terminal in one Ub links to the N-terminal in another Ub, creating linear Ub-chains. Proteins are recognized for degradation by the proteasome if a polyubiquitin chain of at least four Ubs formed at lysine 48 are attached to the substrate protein [118].

Ubiquitination has also been shown to regulate functions beyond degradation. For example, monoubiquitination of histone 2B mediates histone 3 lysine 4 methylation [119,120] and is an integral part in transcriptional activation while monoubiquitination of histone 2A is a repressive mark [121]. Further, monoubiquitination of RNA polymerase II in transcriptional initiation is well established [122]. All clearly connect ubiquitination to transcriptional regulation.

The importance of a functional Ub-proteasome system is illustrated in a number of diseases, for example mutations in the gene encoding the E3 E6-AP causes familial Angelman Syndrome [123] whereas mutations in the gene encoding the E3 Parkin leads to familial Parkinson's disease [124]. Interestingly, E3s have also emerged as important factors for the development of breast cancer [125-127].

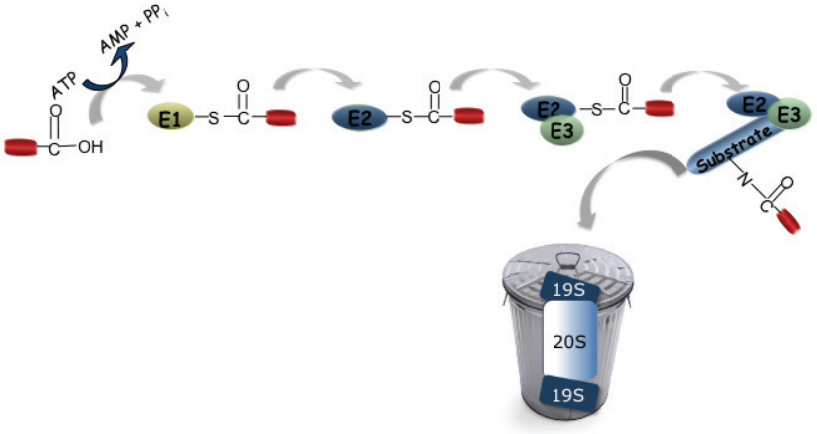

Figure 4. *Ubiquitination and degradation by the 26S proteasome.* Ubiquitin; red. E1; activating enzyme, E2;conjugating enzyme, E3; ubiquitin ligase.

2.7.1 Ubiquitination and transcriptional activation

Several theoretical models, explaining how ubiquitination and proteasomal activity are linked to transcriptional activation, have been proposed [128] [129]. A common feature is that the transcriptional activator is first activated by phosphorylation and then by monoubiquitination, with phosphorylation believed to tag the protein for recognition by the E3s. The function of monoubiquitination is to stabilize the transcriptional activator when bound to DNA [130]. Eventually the activator becomes polyubiquitinated, which targets the activator for degradation. The purpose of polyubiquitination in these models is to allow a "fresh" activator to associate to the promoter [131]. It is important to know that mono- and polyubiquitination can occur at different lysine sites in the activator and by different E3s.

2.7.2 Ubiquitination and transcriptional activation by nuclear receptors

Consistent with the above mentioned models, mutation of monoubiquitination sites in ERα inhibits E2-induced transcriptional activation [132] and transcriptional activation by AR is enhanced by monoubiquitination [133]. Further, polyubiqutination of E2-bound ERα leads to proteasomal degradation while at the same time induces recruitment of RNA polymerase II to some ERα target gene promoters. The question arose as to how degradation of a transcriptional activator could enhance transcriptional activity. It was then shown that ERα binds in a cyclic manner every 45 minutes to its target promoters, as this initiates new rounds of transcription, proteasomal degradation is necessary to clear the promoter so that a "new" ERα can bind and initiate transcription [77,134]. Thus the purpose behind polyubiquitination of a transcriptional activator and subsequent degradation is to allow a "fresh" activator to associate to the promoter. In support of this, the LMP2 subunit of the proteasome interacts with the SRC coactivators, its recruitment to ERα target promoters is crucial for cyclic binding of ERα, RNA polymerase II and SRC-1 to the promoters. It was further demonstrated that proteasomal activity is needed for LMP2-induced activation of ERα target gene promoters [135]. Moreover, the coactivator SRC-3 (AIB1) was shown to be required for degradation of ERα in an E2-dependent manner, importantly, depletion of SRC-3 resulting in decreased promoter occupancy of ERα and RNA polymerase II. SRC-3 was

suggested to regulate degradation of ERα through phosphorylation [136]. As SRC-3 also interacts with the proteasomal subunit SUG-1 [137], it is possible that SRC-3 could recruit SUG-1 in order to mediate degradation of ERα as well as target ERα for degradation through phosphorylation. This model seems, however, to be promoter-selective as inhibition of proteasome activity increases ERα target gene expression if the promoter mainly contains EREs, but decreases target gene expression if the promoter contains multiple transcription factor binding sites [138,139]. It is important to note that a potential role for monoubiquitinated ERα in the cyclic binding to target promoters has not been addressed in the above mentioned studies.

Similar to ERα, proteasomal activity is needed for cyclic recruitment of AR to its target promoters [140]. Transcriptional activation by PR is dependent on proteasomal activity [141]. Additionally, degradation of NR coactivators through the ubiquitin-proteasome pathway has been described [142], adding one more layer of regulation to fine-tune NR-mediated transcription.

Whereas E2 treatment targets ERα for proteasomal degradation [143] and deletion of H12 inhibits E2-induced proteasomal degradation of ERα [77], treatment with Tamoxifen leads to accumulation of ERα protein [134,144,145]. Thus, degradation of ERα could partly be related to its transcriptional activity. However, unliganded ERα is also targeted for proteasomal degradation through interaction with the E3 ligase Carboxyl Terminus of Hsc70-interacting Protein (CHIP) [146]. This suggests that different conformations of ERα affect protein stability through promoting interaction with different E3s.

Importantly, not all NRs seem to require turnover by the ubiquitin-proteasome system to drive gene expression. Such an example is the glucocorticoid receptor where activity is enhanced through histone methylation and RNA polymerase II recruitment upon inhibition of the proteasome [147,148]. Further, a study by Brady *et al.* in 2005 showed that transcriptional activation is more coupled to kinetics than binding [149]. Even though recruitment of the proteasome to certain promoters has been demonstrated, more studies are needed to clarify if this is a general feature in gene regulation [150]. Thus, cyclic recruitment on the promoter for maximum gene expression might be necessary for some, but not all, transcriptional activators.

2.7.3 E3 ubiquitin ligases as nuclear receptor coregulators

The first reports that E3s could work as NR coregulators included the HECT E3s RSP5/RPF1 and E6-AP. The HECT E3 RPF1 enhanced PR and GR-mediated transcriptional activation [151] and E6-AP enhanced transcription mediated by the ligand-bound PR, ERα, AR, GR, thyroid hormone receptor (TR) and retinoic acid receptors (RAR). However, the coactivator function of E6-AP is independent of its ub ligase function but dependent on its ligase domain [152]. E6-AP is recruited to target gene promoters of AR [153] and ERα [134], supporting the theory of E6-AP being a coactivator. Even though the ligase activity of E6-AP was dispensable for its coactivator function, indications for the existence of NR coactivators affecting NR protein stability was demonstrated in assays with mutated coactivator interaction sites in the LBD of ERα which inhibited E2-induced degradation of ERα [77].

Both E3s belonging to the HECT and RING families have been described as NR coregulators. The two most well studied examples are RING E3s that have been associated with oncogenic properties, the Murine double minute clone 2 (Mdm2) and BRCA1.

Mdm2 is most well known for interacting with and targeting the tumor suppressor p53 for degradation. Mdm2 is frequently over-expressed in cancers and a major effort has been put into screening for inhibitors of its binding to p53 [154]. Mdm2 interacts with several NRs including ERα [155], GR and AR [156 157]. While recruitment of Mdm2 to AR target promoters represses AR transcriptional activity [158], Mdm2 functions as an ERα coactivator upon E2 treatment [155]. Mdm2 interacts with the LBD of ERα and in a complex formed together with p53, Mdm2 polyubiquitinates ERα in an E2-dependent manner leading to proteasomal degradation of the receptor [159]. Mdm2 also targets GR for proteasomal degradation [160].

Mutations in BRCA is responsible for approximately 40-85 % of all familial breast cancers [161]. BRCA1 monoubiquitinates the LBD of both agonist and antagonist bound ERα and represses the transcriptional activation of ERα [162-164]. It functions as an inhibitor of ERα –mediated transcription is dependent on its ligase activity [165]. BRCA1's function as a breast cancer suppressor protein, and the importance of its ub

ligase function is highlighted by the fact that all cancer-associated mutations occurring in the RING domain inhibit the ligase activity [166,167]. These mutations result in failure of ubiquitination of ERα in breast cancer cells [163], thereby correlating ERα ubiquitination status to breast cancer. Importantly, it has been shown that mutation of monoubiquitination sites in ERα inhibits E2-induced transcriptional activation [132]. Thus, it is clear that more studies are needed in order to fully understand the function of monoubiquitination in transcriptional activation of ERα. BRCA1 also inhibits the transcriptional activity of PR [168,169].

2.8 RBR E3 UBIQUITIN LIGASES

RING-In-Between-RING (RBR) E3s are a subfamily of the RING family of E3s. In humans they comprise a conserved family with 13 members. The RBR domain is defined by 3 motifs; a N-terminal RING domain (RING1), an In-between-RING domain (IBR) and a C-terminal RING domain (RING2). The RING2 and IBR domains are characteristic for the RBR family while the RING1 resembles the RING domain in other RING family E3s [170-172].

The RBR protein Adriane has been shown to perform ubiquitination in a manner that is somewhat inbetween HECT and RING E3s. Like other RING family E3s, RBRs bind to E2 conjugating enzymes via the RING1 domain. Ub is then transferred to the RING2 domain to form a RING2-Ub, which ubiquitinates the protein substrate via an active-site cysteine similar to the HECT family E3s [173]. In support of this, RBRs have been shown to interact with the E2 conjugating enzyme UBCH7 that lacks an active site for ubiquitination [174]. However, it is not yet clear if this mechanism for ubiquitination is true for all RBR members.

Cellular functions modulated by members of the RBR family includes translation [175] and NF-kB signaling [176,177] and NR signaling [178,179]. Several members of the RBR family have been implicated in disease. For example, loss of Parkin's ligase activity is associated with Parkinson's disease [180], Dorfin degrades SOD1 which causes familial amyotrophic lateral sclerosis [181] and a dominant-negative mutation in ARA54 inhibits transcriptional activation by AR and subsequently AR-induced prostate cancer growth [178].

2.8.1 RNF31 and RBCK1

RING finger 31 (RNF31, also called FLJ10111, PAUL, ZIBRA, HOIP), belongs to the RBR family and was originally identified as a muscle-specific tyrosine kinase receptor interacting protein [182]. It is overexpressed in several types of cancers, including breast cancer [183]. The domain structure of RNF31 includes three zinc ring-finger motifs, a ubiquitin-associated domain (UBA) and a C-terminal RBR domain. UBA domains can bind to both monoubiquitin and polyubiquitin chains, however such functions have not been fully explored for RNF31.

RBCC protein interacting with PKC 1 (RBCK1, also called RNF54, XAP3, HOIL-1) was identified in a yeast two-hybrid screen as a Protein Kinase C (PKC) subtype η [184], β [185], ζ interacting protein and with the capacity to bind to DNA [186]. The C-terminal part of the protein contains the RBR domain, while the N-terminal domain contains an Ubiquitin-like (Ubl) domain and a RanBP2-type zinc finger domain. Ubl domains can interact with the 26 proteasome. However, this has not yet been investigated for RBCK1.

A transcriptional activation function has been mapped to the RBR-domain of RBCK1 in GAL4 assays [187]. In support of this, the splice variant RBCK2 that lacks the RBR domain does not have any transcriptional activation function [188]. However, no endogenous RBCK1 target gene has yet been identified. The RBR domain may also contain a nuclear localization signal as RBCK1 can shuttle between the nucleus and cytoplasm [189], whereas RBCK2 has a cytoplasmic localization [190]. Interaction between RBCK1 and RBCK2 leads to a cytoplasmic localization [190] of RBCK1 and inhibition of the transcriptional activity [188] and ubiquitin ligase activity [191].

RBCK1 and RNF31 have been implicated to have a regulatory function in PKC signaling, however, at the same time PKC signaling seem to regulate their ubiquitin ligase activity [192]. RBCK1 and RNF31 interact to form a linear ubiquitin chain assembly complex (LUBAC) which ubiquitinates activated PKCs. In turn, PKCs inactive the LUBAC ligase activity [192]. Consistent with this, phosphorylation of RBCK1 by PKCβ inhibits RBCK1's ability to autoubiquitinate itself, thereby target

itself for proteasomal degradation [191]. RBCK1 also regulates PKCβ mediated hypertrophy in cardiac myocytes [185]. $PKC\beta_I$ was recently shown to enhance AR-mediated transcription through phosphorylation of histone H3 threonine 6 (H3T6ph), which protects Histone H3 dimethyl lysine 4 (H3K4me2) [193]. It is not yet clear if $PKC\beta_I$ play a similar role in transcriptional activation involving RBCK1.

It was recently shown that another RBR E3 called SHARPIN also takes part in LUBAC and that LUBAC catalyzes the formation of linear ubiquitin chains on NF-kB essential modulator (NEMO/IKKγ). NEMO linear ubiquitination is critical for NF-kB activation that regulates cell survival [194-197]. RBCK1 has also been implicated in antiviral response, interacting with and targeting interferon regulatory factor 3 for proteasomal degradation [198].

3 AIMS OF THE STUDY

Controlled regulation of NR signaling is central to processes such as cellular proliferation, differentiation and metabolism. A major focus of NR research the last decade has centred around how NRs regulate transcription of their target genes. In this respect, the identification of novel NR coregulators has increased our understanding into the tissue-specific response of NRs and how NR signaling becomes deregulated in disease[199]. However, it has become clear that coregulators also affect NR protein stability, leading to the important finding that the ubiquitin-proteasome system can regulate transcriptional activity of NRs[200]. Originally E3s were thought to only target proteins for degradation by the proteasome. However, in the last decade this view has been extended and it is now clear that the consequence of interacting with an E3 is far more complex. Although a few NR-interacting E3s that act as coregulators have been described, little is known about their molecular functions beyond potential turnover of NRs. Therefore, the general aim of this thesis was to identify and characterize two E3s belonging to the RBR subclass that are important, novel modulators of NR signaling.

The specific aims were:

I. To identify and characterize novel coregulatory functions of RBR ubiquitin ligases in nuclear receptor signaling (Paper I, II, III).

II. To identify and characterize a novel DAX-1 coregulator (Paper I).

III. To identify and characterize novel regulators of proliferative ERα signaling in breast cancer cells (Paper II, III).

IV. To investigate the molecular mechanism behind potential regulation of ERα gene expression by RBCK1 (Paper III).

4 RESULTS AND DISCUSSION

4.1 PAPER I

In order to identify novel DAX-1 interacting proteins, a yeast-two hybrid screen was performed with DAX-1 as bait. We found that RNF31, an RBR ubiquitin ligase, interacted with DAX-1, this interaction was further confirmed in co-immunoprecipitation and GST-pull down assays. The DAX-1 domain responsible for interaction was mapped to the third LXXLL-repeat in the N-terminal domain. Consistent with this, small heterodimer partner (SHP), also containing this motif, was the only additional NR found to interact with RNF31. The RNF31 domain required for DAX-1 interaction was mapped to the central ubiquitin-associated (UBA) domain.

RNF31 expression was found to overlap with DAX-1 expression in steroidogenic tissues such as the outer layer of the adrenal cortex and testis. Interestingly, RNF31 was expressed in cell lines that do not express DAX-1, indicating a function beyond regulating DAX-1 activity. The human adrenocortical cell line H295R was chosen as a model system to investigate the RNF31 and DAX-1 complex as this is the only human steroidogenic cell line known to express DAX-1. DAX-1 and RNF31 co-localized both in the nucleus and cytoplasm. Consistent with the presence of an RBR ubiquitin ligase domain in RNF31, RNF31 mediated mono-ubiquitination in the LBD of DAX-1 in an RBR-dependent manner. To our knowledge, this is the first post-transcriptional modification described for DAX-1. RNF31 overexpression and siRNA targeting RNF31 demonstrated that RNF31 stabilized DAX-1 protein levels, indicating a non-proteolytic function of RNF31.

In order to investigate a role for RNF31 in DAX-1 target gene expression, we measured promoter reporter activity and mRNA and protein expression of DAX-1 repressed targets upon depletion of RNF31. StAR and CYP19 promoter activity, mRNA and protein expression were significantly increased in siRNF31 treated cells. Further, chromatin immunoprecipitation (ChIP) assays revealed that the presence of RNF31 at the CYP19 and StAR promoters was necessary for promoter occupancy by DAX-1 and the corepressor SMRT. This suggests that RNF31, DAX-1 and SMRT form a corepressor complex *in vivo* on CYP19 and StAR promoters. Recruitment of the ternary corepressor complex was depending on the promoter occupancy of SF-1.

It is possible that RNF31 has a stabilizing role in promoter occupancy by DAX-1 and SMRT. RNF31-dependent monoubiquitination of DAX-1 is in support of the models of how DNA-associated transcription factors are stabilized through monoubiquitination. It would be of interest to further investigate the presence of monoubiquitination of DAX-1 on target promoters and how this modification alter DAX-1 transcriptional ability. To the author's best knowledge, presence of monoubiquitinated NRs on the actual target promoters has not yet been determined. Monoubiquitin fusion proteins have successfully been used when assaying the consequence of monoubiquitination for protein function[201]. Employing DAX-1-Ubmono with mutated lysine sites for Ub-chain formation in Ub, and then measure global expression profiles, would provide insight into if this modification generally enhances corepressor function of DAX-1 or just regulates a subset of DAX-1 target genes.

The RNF31-interacting NR SHP belongs to the same subgroup of NRs as DAX-1 and can heterodimerize with DAX-1 [202]. However, a function of SHP in the adrenal gland remains to be investigated. Future studies should address a potential role for 1) SHP in RNF31-mediated repression of steroidogenesis in the adrenal gland and 2) RNF31 in SHP-expressing tissues such as the liver.

As DAX-1 is expressed in other tissues than the adrenal gland, such as mammary tissue, it would be interesting to investigate if RNF31 also modulates DAX-1 function in these tissues. Further, it would be interesting to compare the phenotype of a RNF31 knock-out mouse model to the phenotype of SF-1 knock-out mice and AHC patients.

In summary, we have identified that RNF31 is a novel DAX-1 coregulator necessary for successful transcriptional repression of SF-1 in steroidogenesis.

4.2 PAPER II

The purpose of this study was to identify novel regulators of proliferative ERα signaling in breast cancer cells.

Three initial observations lead to the hypothesis that RBCK1 could regulate the proliferation of ERα-positive breast cancer cells. Firstly, through a bioinformatics approach, we found RBCK1 to be homologous to E3 ubiquitin ligases that regulate cell cycle progression. Secondly, RBCK1 is known to interact with Protein Kinase C subtypes implicated in estrogen signaling. Finally, using the Oncomine database, we found RBCK1 mRNA to be elevated in breast cancer and specifically upregulated in ERα-positive breast cancer.

We confirmed that RBCK1 is expressed in the human breast cancer cell lines MCF-7 and T-47D. Further, cells depleted of RBCK1 had reduced cell viability due to cell cycle arrest. Specifically, siRBCK1 inhibited estrogen-induced S phase progression and induced an estrogen-independent arrest in the G2/M phase. In line with the effect of estrogen on cell cycle progression being largely mediated by ERα, both ERα mRNA and protein levels were significantly decreased in RBCK1 depleted cells. Further, mRNA levels of ERα target genes Cyclin D1 and c-myc, important for progression from the G1 to S phase, were decreased upon RBCK1 depletion. Thus, RBCK1 depletion impairs proliferative ERα signaling in breast cancer.

The estrogen-independent cell cycle arrest upon RBCK1 depletion was correlated with downregulation of Cyclin B1. We hypothesized that RBCK1 might support transcription of Cyclin B1 through recruitment to the Cyclin B1 promoter region as the mRNA expression of Cyclin B1 was down-regulated by RBCK1. We could however not observe any recruitment of RBCK1 to the proximal Cyclin B1 promoter. This may be due to that RBCK1 regulates Cyclin B1 expression via unknown promoters or distal enhancer elements. As no response element for RBCK1 is known we were unable to scan Cyclin B potential regulatory regions for potentially regulatory regions using a bioinformatic approach. Downregulation of Cyclin B1 could also be a secondary effect of RBCK1 down regulation. RBCK1 might affect signaling cascade(s) including transcriptional regulator(s) that regulate Cyclin B1. Overall, this highlighted one of the

problems with using siRNA approaches; that it is difficult to distinguish between primary/direct and secondary/indirect effects.

As ERα mRNA levels were decreased upon RBCK1 depletion, we next determined if RBCK1 might have a function in regulating ERα gene transcription. Indeed, RBCK1 was recruited to promoter B, stimulating expression from both RBCK1 promoters A and B. This is not surprising, since the distance between the ERα promoters A and B is small. To our knowledge this is the first report of an endogenous RBCK1-target gene.

A positive correlation between RBCK1 and ERα expression was demonstrated in a small set of clinical breast cancer samples. We were able to confirm this correlation in larger data set from publically available clinical breast cancer microarray data using a Pearson correlation test. It is important to note that the R square value was approximately 0.3 in all datasets meaning that RBCK1 can be held responsible for 30% of the ERα expression in breast cancer. Thus, it is likely that several mechanisms contribute to the increased levels of ERα in breast cancer. This is expected as several transcription factors have been described to regulate the ERα expression, as discussed in the section *2.4.1.1*.

In conclusion, we found that RBCK1 promotes cell cycle progression from both the G1 to S phase, via promoting ERα expression and thereby ERα signaling, as well as the G2/M phase, possibly by modulation of Cyclin B1 expression.

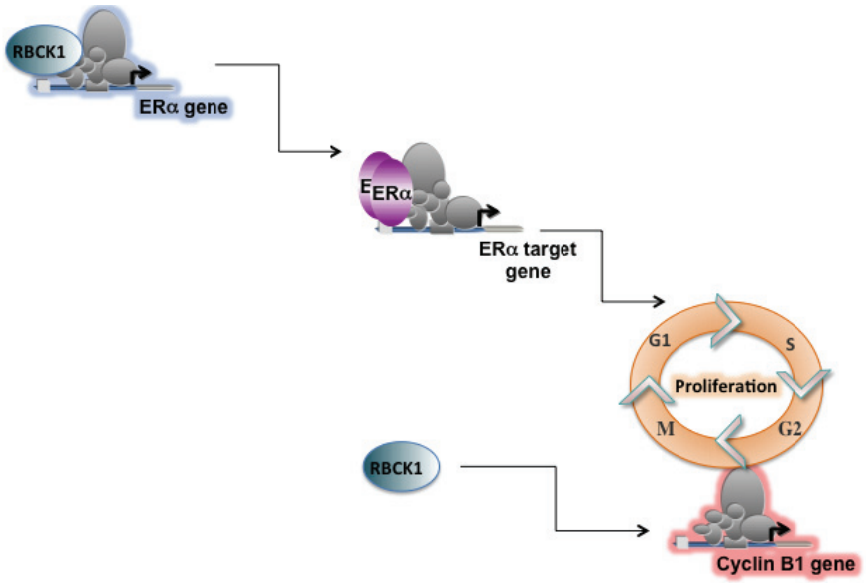

Figure 5. *Mechanism of RBCK1 action contributing to breast cancer cell proliferation.*

4.3 PAPER III

Modulation of ERα signaling by other nuclear receptors, ligands, coregulators, post-transcriptional modifications and cell signaling has been well described [81]. However, studies investigating regulation of ERα gene expression are limited. The aim of paper III was to investigate the molecular mechanism by which RBCK1 regulates ERα expression in breast cancer cells.

From the results in paper II, we hypothesized that RBCK1 works as a transcriptional activator of ERα promoters A and B in breast cancer cells. We confirm here in this study using an ERα promoter A-B reporter construct, as well as measuring an increase in endogenous ERα mRNA and protein levels upon RBCK1 overexpression in MCF-7 breast cancer cells, that RBCK1 can upregulate ERα expression.

Through sequential ChIP we observed that RBCK1 and ERα co-occupy a previously confirmed ER binding region in the ERα promoter B. ERα has also been shown to associate with upstream enhancers in the ERα promoter. Importantly, we could not detect any recruitment of RBCK1 to these enhancers. We demonstrated that interaction between endogenous RBCK1 and ERα and ERα recruitment to the promoter was necessary for RBCK1 association to the promoter. On the contrary, RBCK1 recruitment was not necessary for association of ERα to the ERα promoter B. Overexpression of RBCK1 together with ERα enhanced ERα promoter reporter activity approximately 10-fold compared to transfection with RBCK1 or ERα alone. Altogether suggesting an ERα coactivator function of RBCK1. Alternatively confirmed ERα corepressors such as Sin3A [52] are recruited by ERα to the promoter in the absence of RBCK1, resulting in repression of ERα gene expression.

We found that RBCK1-mediated transcriptional activation of the ERα promoter was dependent on the RING2 domain. This is consistent with a previous study showing that the rat homologue of RBCK1 can activate transcription in GAL4 assays via its C-terminal RBR domain [187].

In support of the model suggested for RBR-mediated ubiquitination, where Ub is transferred from RING1 to RING2 leaving the IBR domain available for interaction

with the protein substrate, RBCK1 interacted with ERα via the IBR domain. As deletion of RING2 severely decreased the transcriptional activation by RBCK1, it is possible that ubiquitination mediated by RBCK1 is an important function in its transcriptional activation. Recent publication makes it possible to predict active sites necessary for Ub ligase acitvity in RBCK1 [173]. Future studies should include point mutations of such sites in order to determine if RBCK1 coactivator and Ub ligase activity colocalizes.

As the combination of E2 conjugating enzymes and E3s mediate substrate specificity it would be of interest to identify the potential E2 interacting with RBCK1. Two E2s have been found to interact with RBR ubiquitin ligases, UBCH7 and 8[203]. Interestingly, UBCH7 is recruited to ERα target promoters[204]. Thus, it is possible that 1) UBCH7 interacts with RBCK1 and 2) UBCH7 is part of the RBCK1-ERα complex on the ERα promoter B.

Interestingly, the RBCK1-interacting protein Protein Kinase C beta 1 (PKCβ1) was recently implicated in regulation of AR target gene activation by phosphorylation of histone 3 threonine 6 (H3T6ph), that in turn protects dimethylation of lysine 4 (H3K4me2) [193]. This lead to our hypothesis that RBCK1 might support ERα transcriptional activation through interaction with PKCβ1, that in turn modifies H3. Indeed RBCK1 and PKCβ1 co-occupied the ERα promoter B. Moreover, siRBCK1 treatment resulted in reduced promoter recruitment of PKCβ1 as well as reduced levels of H3T6ph and H3K4me2. As H3 lysine 4 methylation can be mediated by Histone 2B ubiquitination it would be interesting to investigate if RBCK1, considering its Ub ligase domain, can modulate this modification.

Even though RBCK1 promoter occupancy was associated with a permissive chromatin environment it would be interesting to determine status of markers for transcriptional initiation such TFIID and B or phosphorylation of the C-terminal domain (CTD) of RNA polymerase II upon depletion of RBCK1 promoter occupancy in order to more specifically determine if RBCK1 is important for recruitment of GTFs to the ERα promoter.

Consistent with the transcriptional function of PKCβ1 being dependent on its kinase function[193], we observed that ERα mRNA expression was decreased upon inhibition of the kinase activity. Subsequently, ERα protein levels and target gene expression of Cyclin D1 and c-myc were also decreased. To directly relate this decrease in ERα expression to PKCβ1-dependent phosphorylation of H3, we have identified that inhibition of the kinase activity leads to decreased H3T6 phosphorylation in the ERα promoter B (*Gustafsson Sheppard unpublished data*). PKCβ1 has previously been shown to affect Cyclin D1 expression by an unknown mechanism[205]. As Cyclin D1 is an ERα target gene, it is possible that the effect of PKCβ1 on ERα expression, reported here is to mediate the previously described observation that PKCβ1 modulates Cyclin D1.

We could not find that the presence of E2 affected RBCK1- ERα interactions or transcriptional regulation of the ERα promoter. In support of a possible ligand-independent function of the ERα-RBCK1 complex, we have determined that 1) Tamoxifen does not influence ERα-RBCK1 complex and 2) the non-ligand dependent AF-1 domain of ERα is critical for interaction with RBCK1. (*Gustafsson Sheppard unpublished data*). More experiments are needed to fully evaluate the possible influence by pure ERα antagonists on the RBCK1- ERα complex and function in breast cancer cells.

In order to investigate if RBCKI and PKCβ1 are recruited to a subset of ERα target gene promoters or are more general coregulators for ERα, RBCK1- PKCβ1 recruitment was assayed at randomly selected ERα binding regions**.** Recruitment was confirmed at all selected binding regions (*Gustafsson Sheppard unpublished data*). Conversely, we could not find any recruitment of RBCK1- PKCβ1 to ERα binding sites in enhancers upstream the ERα gene. Since only 5% of ERα binding sites are located within 5kb from the transcriptional start site [206], it could be that RBCK1- PKCβ1 are recruited to a subset of ERα binding sites, possibly the ones located in promoter regions. Importantly, the AF-1 domain of ERα is needed for interaction with RBCK1 *(Gustafsson Sheppard unpublished data*). Whether RBCK1 and PKCβ1 may be recruited to a subset of ERα target gene promoters possibly regulated through AF-1 activity should be focus of future studies.

Taken together, our data suggests a novel ERα coactivator function of RBCK1 in which RBCK1 supports transcriptional activation through enabling PKCβ1 promoter occupancy and subsequently PKCβ1-dependent histone modifications. Thus, a complex of ERα/RBCK1/PKCβ1 on the ERα promoter B would drive ERα gene expression, thereby promoting proliferative ERα signaling in breast cancer cells. This mechanism is distinct from the described mechanism by which proteolysis promotes the transcriptional activation function of ERα.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The general aim of this thesis was to identify and characterize two E3 Ub ligaess, RNF31 and RBCK1, that belong to the RBR subclass that modulate NR signaling. Originally E3s were thought to only target proteins for degradation by the proteasome. However, in the last decade this view has been extended and it is now clear that the consequence of interacting with a E3 is far more complex, of great interest is the involvement of the Ub-proteasome system in transcriptional activation[200]. However, only a few NR-interacting E3s that are able to act as coregulators have been described, with little known regarding their molecular functions beyond the potential turnover of NRs [207]. Our studies presented in this thesis investigate the mechanisms behind RNF31 and RBCK1's ability to modulate transcription through coregulatory functions. As both E3s are recruited to NR target gene promoters and are necessary for the formation of transcriptional complexes associated with repression (Paper I) or activation (Paper III), our novel findings clearly strengthen a coregulatory, non-proteolytic function for E3s in NR signaling.

A major finding in this thesis is that RBCK1 supports proliferation of ERα-positive breast cancer cells via transcriptional regulation of ERα (Paper II, III). Considering the important role of ERα signaling in development and progression of a majority of breast cancers, it is somewhat surprising that the regulation of ERα gene expression has not been more characterized. Since cell-type specific promoter usage for ERα expression occurs[47,48], identification and characterization of promoter specific transcription factors may aid in development of novel cell-type specific therapies manipulating ERα signaling in breast cancers. Furthermore, increased levels of ERα, independent of the presence of estrogen, result in development of ductal carcinoma in mice [208] and increased intracellular levels of ERα results in ligand-independent activation of the receptor [56,57]. Additionally, ERα can become activated by growth factors [27]. Thus, the intracellular level of ERα might be an important factor in breast cancer development as well as resistance to treatment. Therefore, targeting ERα in a way that eliminates receptor expression in breast cancer is of high interest. Targeting promoter-specific regulators of ERα gene expression, such as RBCK1 and potentially PKCβ1, might provide a novel approach, in addition to SERDs, to down-regulate the receptor in breast

cancer. Particularly as usage of the ERα promoter B, that according to our studies are regulated by RBCK1 and PKCβ1, seems to be specific for breast cancer [59]. However, as ERα mRNA B and A are expressed in both mammary tissue and ovaries[48], further studies investigating potential functions of RBCK1 and PKCβ1 in regulation of ERα expression in the ovaries are needed.

Several issues have to be addressed in order to evaluate the potential of RBCK1 as a therapeutic target in breast cancer. Importantly, more studies are needed to fully dissect the specificity in RBCK1-mediated regulation of ERα in molecular, cellular and mice models. For example, RBCK1 have has functions beyond ERα. Thus, RBCK1 expression and function in other tissues has to be determined in order to avoid unwanted effects when targeting the protein. Further, a suitable assay has to be developed in order to measure RBCK1 activity or function in order to perform a high-throughput screen for novel compounds exhibiting RBCK1 inhibitory properties. The last 5 years, small molecules disrupting protein-protein interactions have developed as successful therapeutic agents [209]. Thus, identifying molecules that disrupt RBCK1- ERα interactions would provide a strategy to inhibit RBCK1-mediated upregulation of the ERα promoter in breast cancer. Sensitive measurement of RBCK1 and ERα interactions in the presence of various compounds could be performed in a protein-fragment complementation (PCA) assay[210] with RBCK1 and ERα fused to complementary parts of a fluorescent protein. Lastly, investigating a functional role for RBCK1 by employing hits from high-throughput screenings in a mouse model of breast cancer such as MMTV-PyMT [211] will provide valuable information into modulation of breast cancerogenesis by RBCK1.

Interestingly, drugs targeting PKCβI and PKCβII such as Ruboxistaurin are under current clinical trial for the treatment of diabetic peripheral retinopathy [212] indicating that PKCβ1 is a potential drug target. Whether PKCβ1 expression or kinase activity correlates with ERα expression in samples from breast cancer patients awaits further clarification.

Our results show that RBCK1 and ERα mRNA expression positively correlates in clinical breast cancer samples. Further investigation into the expression of RBCK1 and PKCβ1 in 1) different stages of breast cancer, 2) short- and long-term breast cancer

survival, 3) response to endocrine treatment, will provide insight into a possible prognostic value of assessing RBCK1 or PKCβ1 expression in breast cancer.

Future studies should investigate if RBCKI and RNF31 display gene promoter specific recruitment and if they are general coregulators for their interacting NRs**.** As RNF31 and RBCK1 interact, forming the Ub ligase complex LUBAC[195], it is possible that RNF31 and RBCK1 modulate eachothers functions as transcriptional coregulators in steroidogenesis and breast cancer proliferation. Future studies addressing these issues should also include SHARPIN, another RBR ligase, recently reported to be part of LUBAC.

The studies presented in this thesis are focused on further elucidating the coregulatory functions of E3s in NR signaling. However, we have only begun to characterize RNF31's role in modulating steroidogenesis and RBCK1's ability to drive ERα-positive breast cancer cell proliferation. Many aspects in characterizing these functions remain unexplored and so many exciting studies await in order to fully understand their mechanisms of action.

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